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THE INFLUENCE OF INORGANIC IONS AND ACCLIMATION SALINITY ON HEMOCYANIN-OXYGEN BINDING IN THE BLUE CRAB *CALLINECTES SAPIDUS*

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Abstract

The effects of salinity changes on the oxygen binding properties of hemocyanin have been examined in the blue crab *Callinectes sapidus*. Oxygen affinity increases measurably with increases in Ca^{+2}, Mg^{+2} or Na^{+} but, within the physiological range, not K^{+}. Unlike its large and specific influence on other arthropod hemocyanins, Cl^{-} has little or no effect. Ca^{+2}, alone and in physiological concentrations, restores oxygen affinity to the level observed in a complete physiological saline. Ca^{+2} also restores the Bohr shift to the physiological level. Physiological variation in inorganic ions (or pH) does not affect the cooperativity of oxygen binding.

Physiological variation in Ca^{+2} explains only a minor fraction of the actual change in oxygen affinity that accompanies acclimation to a new salinity. The acclimation, which occurs within 8 days, requires a non-dialyzable factor in the blood. The non-dialyzability of the factor and the previous reports of a large increase in blood protein levels at low salinity might implicate an intrinsic change in the hemocyanin molecule with salinity, a suggestion that is also supported by apparent differences in the subunit composition of the molecule in the high and low salinity acclimated crabs. Using paired observations on the same individuals before and after acclimation, however, we were able to demonstrate relatively small changes in hemocyanin concentration. Moreover, the relationship between the differences in subunit composition and HcO_2 binding is uncertain. The identity of the factor remains unknown.

Introduction

The influence of the ionic environment on the oxygenation properties of the hemocyanins (Hcs) has been recognized for many years. Among the crustaceans, HcO_2 affinity increases with the addition of inorganic ions and decreases with the addition of H^{+} (Redfield, 1933; Larimer and Riggs, 1964; Chantler *et al.*, 1973; Truchot, 1975; Mangum and Towle, 1977; Brouwer *et al.*, 1978). In many euryhaline species the opposite responses to pH and inorganic ions are especially important because they actually occur *in vivo*, with the net effect of stabilizing the performance of the HcO_2 transport system (Truchot, 1975; Weiland and Mangum, 1975). A detailed theoretical analysis of the opposite effects of Mg^{+2} and H^{+} suggests that the mechanism is competition at the same ion-binding sites, a few of which are linked to O_2 binding sites (Arisaka and Van Holde, 1979).

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Abbreviation: Hc, hemocyanin.

The influence of physiological changes in inorganic ions on other HcO_2 binding properties has not been extensively investigated in the crustaceans; the available information suggests that physiological changes in blood salts have little or no effect on the Bohr shift (Truchot, 1975) or cooperativity (Miller and Van Holde, 1981).

The influences of the various inorganic ions found in the blood are not equal. On a molar basis, divalent cations have a greater effect on HcO_2 affinity than any of the monovalent ions (*e.g.* Truchot, 1975; Brouwer *et al.*, 1978). On a molar basis, however, physiological changes in organic ions are also unequal. Divalent cations are both less abundant and, in most species, regulated far more strongly than Na^+ and Cl^-. Should they prove to be effectors, the monovalent ions could be more important (Mangum, 1981).

Although monovalent cations have not been widely examined, the effects of Cl^- and HcO_2 affinity have been investigated in a number of arthropods, with surprisingly different conclusions. A specific Cl^- effect was first demonstrated in the chelicerate *Limulus* by Sullivan *et al.* (1974) and later elucidated by Brouwer *et al.* (1977). The chelicerate and crustacean hemocyanins have many structural and functional dissimilarities, however. Of greater relevance here, a clear effect of total salinity was found in the brachyuran *Callinectes sapidus* (Mangum and Towle, 1977), which maintains a very nearly homeostatic condition of the divalent cations in its blood (Colvocorresses *et al.*, 1974). The specificity of a quite large Cl^- effect was demonstrated in the dendrobranchiate crustacean *Penaeus setifer* by Brouwer *et al.* (1978). Although the specificity of the individual ions is not yet known, clear effects of both total salinity and mixtures of Cl^- salts have also been found in other crustaceans such as the thalassinid shrimp (Miller and Van Holde, 1981).

In contrast, Truchot (1975) reported that in the brachyuran *Carcinus maenas*, a member of the same family as *C. sapidus*, the effect of NaCl on HcO_2 affinity is very small, non-specific and, at least within the physiological range, opposite to the salt effects observed in other crustaceans. Using constants calculated from curves describing the response to each of several inorganic salts, he concluded that the response to total salinity can be explained almost in full by changes in the divalent cations, which are regulated less perfectly in *C. maenas* than in *C. sapidus*.

With one exception (Miller and Van Holde, 1981 and pers. comm.), the protocols used in experiments on individual ionic effects have involved first removing salts by dialysis, in some instances at high pH and in the presence of EDTA, and then adding salts in the desired ratios. Even at physiological pH and without EDTA, this procedure causes at least some dissociation of the native Hc polymer to its monomeric subunits. Following complete dissociation and the readdition of critical ions the monomers reassemble again, but often to polymers smaller than the native molecule and with an "incorrect" subunit composition that alters O_2 affinity and its ion dependence (*e.g.* Jeffrey and Treacy, 1980). Thus it is not entirely clear that the ion sensitivities of the native arthropod hemocyanins are as diverse as implied by the literature.

The suggestion that divalent cations are responsible for the influence of total salinity (Truchot, 1975) seemed difficult to reconcile with the observations on *C. sapidus*. Either the divalent cation sensitivity of HcO_2 affinity is much greater in this strong regulator, or other factors are also involved. In addtion, an experiment performed by Mangum and Towle (1977) seemed difficult to reconcile with the important finding that the L-lactate produced during hypoxia raises HcO_2 affinity (Truchot, 1980), an effect that has now been demonstrated in *C. sapidus* as well as several other decapods (Johnson and Becker, 1981; Booth *et al.*, 1982; Graham *et al.*, 1982). Mangum and Towle (1977; Fig. 3) illustrated the responses of the HcO_2

transport system to the estuarine environment by comparing animals acclimatized to dilute and also moderately hypoxic waters with different animals acclimatized to more saline and also normoxic waters. Since the difference in HcO_2 affinity (3–4 mm Hg) could be reproduced *exactly* by altering the total salinity and pH of the blood of an individual animal, they attributed it to pH and salinity *per se*. According to Johnson and Becker (1981 and pers. comm.), however, even the small amounts of lactate produced during hypoxia should raise HcO_2 affinity by several fold more than observed.

We have investigated the basis of the salinity effect on HcO_2 affinity, and also other respiratory properties of the blood, in *Callinectes sapidus* Rathbun, using 1) procedures that do not alter the size or the subunit composition of the native polymer, 2) preparations stripped of dialyzable factors such as lactate, and 3) in an *in vivo* experiment, salinity alone as a stimulus of the change in the same individuals. We have identified the specific and the physiologically important inorganic ion effectors, and we have demonstrated an additional factor that dominates the response and thus explains several of the apparent contradictions in the literature.

Materials and Methods

Collection and maintenance of animals

The effects of inorganic ions on HcO_2 binding were first examined using blood of crabs purchased during March, April and May from large commercial suppliers in the lower Chesapeake Bay. Animals were held for less than 24 h in recirculating water at 18‰ and 21–23°C, a period during which a temperature change has no effect (Mauro and Mangum, 1982). In most experiments, the blood of 27–36 individuals was pooled and the pool used for a related set of experiments; an exception in which paired observations were made on the same individuals is described in detail below. The animals were adult males in intermolt stage C ranging in carapace length from 6.0 to 7.3 cm.

Preparation of hemocyanin

Blood was extracted from the infrabranchial sinuses of each walking leg, allowed to clot in a tissue homogenizer, and then the clot was broken and separated from the serum by centrifugation. Ten ml aliquots of the pooled sera were dialyzed at 5°C against 1 l of the test solution (see below), buffered with 0.05 *M* Tris Maleate. The medium was changed after 24 h and the dialysis continued for an additional 24 h. The preparation was centrifuged again prior to the O_2 binding measurements.

Preparation of test solutions

Since our purposes were to investigate the effects of maximum physiological changes in inorganic ions and to distinguish clearly the effective from the ineffective ions in the blood, the entire physiological range found in nature was examined (Mangum and Amende, 1972; Lynch *et al.*, 1973; Colvocoresses *et al.*, 1974). We should emphasize that the physiological range is estimated from acute measurements (*i.e.*, made on freshly collected animals), and therefore they exaggerate the average physiological variation. This point is considered further in the Discussion.

The test solutions were prepared so that the activity of each ion in the single salt solutions and in the mixtures of two or three salts closely approximates the value in a graded series of physiological salines containing all of the major inorganic

TABLE I

Ionic composition of the complete physiological salines against which C. sapidus *blood was dialyzed*[1]

	Concentration (meq/l)							
	Solution 1		Solution 2		Solution 3		Solution 4	
Ions	Total	Free	Total	Free	Total	Free	Total	Free
Na^+	138	90	278	181	410	270	546	359
K^+	4	2.5	8	5.0	12	7.5	16	10.0
Ca^{+2}	15	2.6	25	4.8	35	7.0	45	9.2
Mg^{+2}	15	1.1	25	1.8	35	2.5	45	3.3
Cl^-	150	94	300	190	450	283	600	377
HO_3^-	2	1.2	2	1.2	2	1.2	2	1.2
SO_4	20	1.0	30	1.4	40	2.8	50	3.3
Total Salinity	342	194	664	386	984	574	1304	764

[1] The concentration of the free ions in the single and multiple salt solutions approximate the concentration of the free ions in the complete salines. Solutions were buffered with 0.05 *M* Tris Maleate.

ions found *in vivo*. The activity coefficients, given in detail by Mason (1982), were taken from Robinson and Stokes (1970) and from Pytkowicz *et al.* (1975). The composition of the series of physiological salines is given in Table I. The concentrations of free Na^+, K^+, Ca^{+2}, Mg^{+2} and Cl^- in the dialysate, or the product of total concentration and the activity coefficient, were ascertained by direct measurement with ion selective electrodes, using 1:99 dilutions of buffered, stirred samples. Further details of this procedure are described by Graham *et al.* (1982).

Oxygen binding measurements

Oxygen binding measurements were initiated within 30 min of completing the dialysis, using physiological concentrations of the protein. The sample was allowed to equilibrate for 10 min at atmospheric pressure with each of 4–7 humidified mixtures of N_2 (ultra high purity, scrubbed further in a 120×3 cm column of Ridox) and either O_2 (purity 99.5%) or air (scrubbed of CO_2 with KOH). The mixtures were prepared with a Wösthoff gas mixing pump. During the equilibration period, the vessels were immersed in a water bath (25°C), and the samples mixed. At each PO_2, changes in absorbance at 335 nm (1 mm light path) were noted with a Bausch and Lomb Spectronic 20 colorimeter.

Measurements of pH were made at 0 and 100% HcO_2. A Fisher Accumet 520 pH meter and a Radiometer electrode were used. The pH of the oxygenated and deoxygenated Hc preparations never differed by more than 0.02 pH units.

Two to four replicate measurements were performed on each preparation, and the data treated as a homogeneous sample. P_{50} and n_{50} values were determined from logarithmic regression lines fit to Hill plots of the pooled data in the range 15 to 85% HcO_2. The significance of differences between the values for n_{50} were estimated from the 95% confidence intervals around the slope and, for P_{50}, from 95% confidence belts around the regression lines.

Experimental design

The experiments testing the effects of different concentrations of individual ions and salt mixtures on HcO_2 binding at a common pH were designed so that the

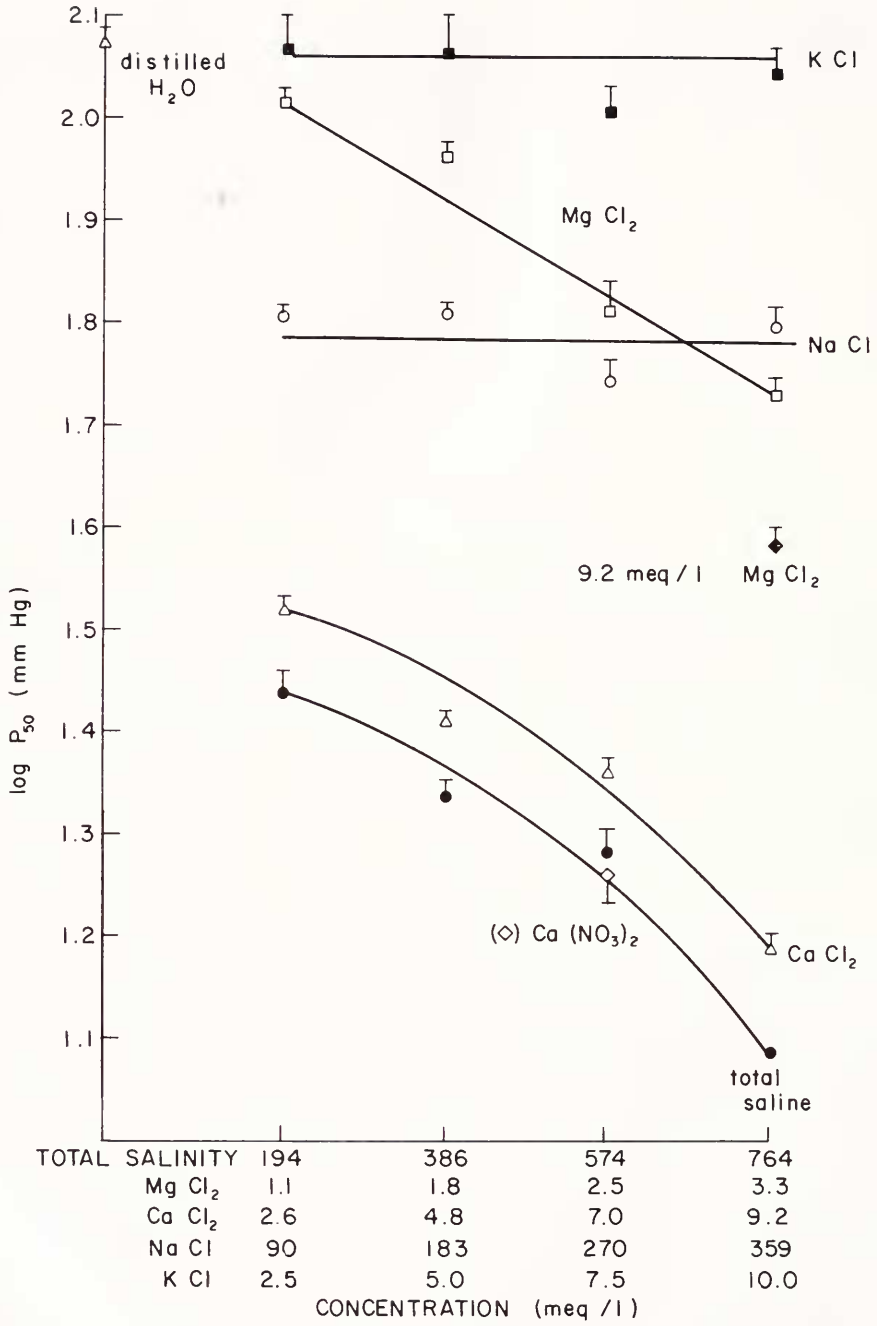

FIGURE 1. Effects of a complete physiological saline and of single inorganic salts on O_2 affinity (P_{50}) of *C. sapidus* Hc in 0.05 *M* Tris Maleate (pH 7.50 ± 0.22) at 25°C. Free ion concentration is given. (◇) 7.0 meq/l Ca $(NO_3)_2$. Bars show 95% confidence interval.

response of P_{50} and n_{50} to total salinity is the control, and the response to the test ions corresponding to a particular salinity is the experiment. For example, in Figure 1 the point at 194 meq/l salinity is the control for 1.1 meq/l $MgCl_2$, 2.6 meq/l

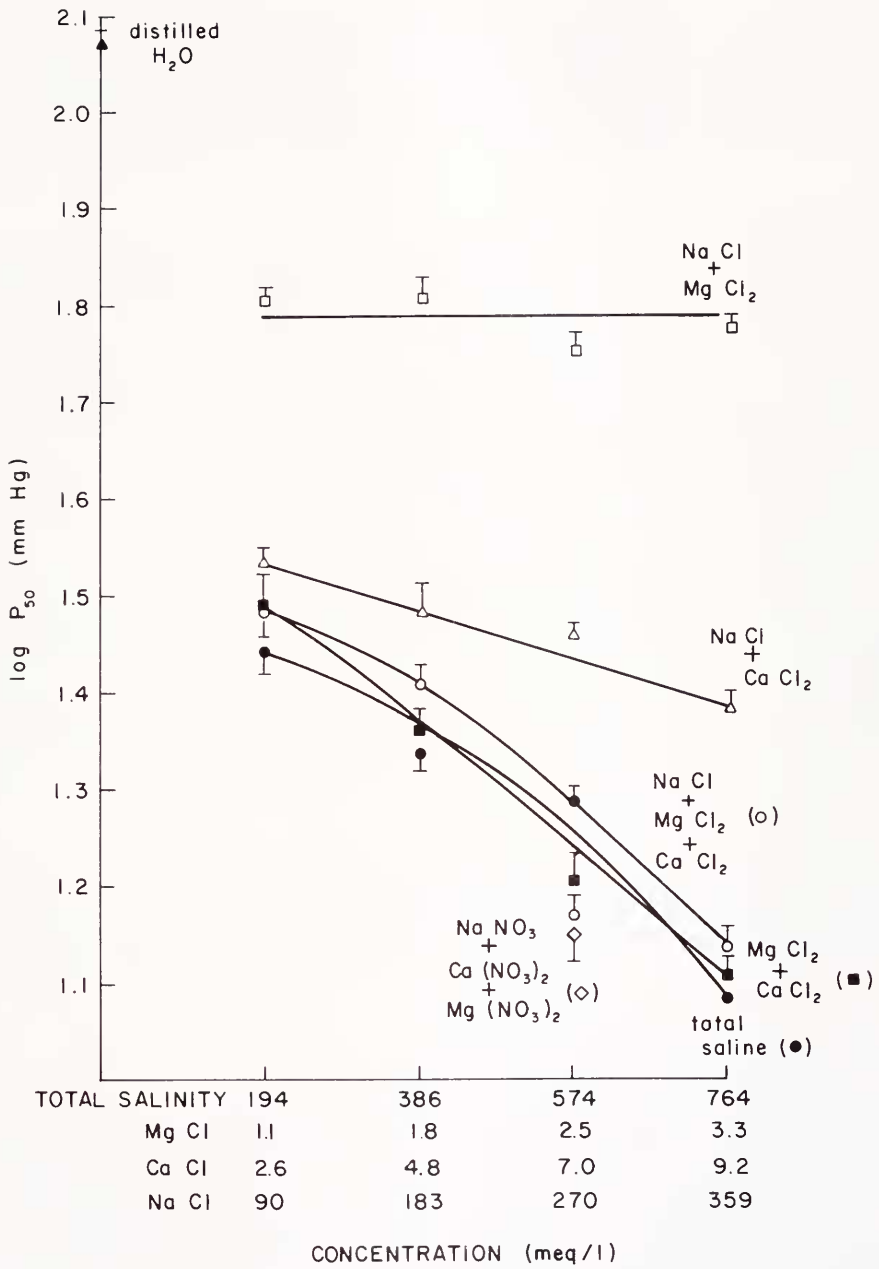

FIGURE 2. Effects of mixtures of inorganic salts on O_2 affinity (P_{50}) of *C. sapidus* Hc in 0.05 *M* Tris Maleate (pH 7.50 ± 0.02) at 25°C. Free ion concentration is given. Curve for complete physiological saline (●) is reproduced from Figure 1. (◇) 270 meq/l $NaNO_3$, 7.0 meq/l Ca $(NO_3)_2$, 215 meq/l Mg $(NO_3)_2$. Bars show 95% confidence interval.

$CaCl_2$, 90 meq/l NaCl and 2.5 meq/l KCl. A different pool of blood was used at each of the four sets of concentrations shown in Figures 1 and 2. Therefore, each point obtained at the different concentrations and thus connected by the curves represents a different pool of blood.

The experiments testing the effects of critical ions and salt mixtures on the Bohr shift were designed so that the response of the factor $\Delta \log P_{50}/\Delta pH$ to total salinity is the control and the response to single salts or a mixture of two salts is the experiment. The data obtained at each particular concentration and thus the points connected by the curves in Figures 3–6 represent the same pool of blood.

Acclimation to high and low salinity

Adult, intermolt males were purchased in June 1981 from a commercial supplier who had caught them in the upper York River estuary (0–3‰ salinity, measured with a Yellow Springs Instrument Co. conductivity meter) within the previous 6 h. Adult, intermolt males were also captured in pots by the authors in inlets of the Atlantic Ocean near Wachapreague, Virginia (34‰). While we cannot exclude the possibility of exchange between the two populations, the distance would require a travel period of several weeks. Each group was held for less than 12 h in aerated, natural water (21–23°C), the low salinity group at 5–8‰ (York River estuary water) and the high salinity group at 35‰ (Wachapreague Inlet water). Two ml of blood were taken from each crab; the crab was then transferred to the alternative salinity, held there for 8 days without feeding, and sampled again.

Half of the blood from each crab taken prior to and following the transfer was dialyzed against a complete physiological saline representing high salinity, and the other half was dialyzed against a saline representing low salinity. Oxygen binding measurements were made as described above and the Hc concentration estimated from the absorbance of [HcO_2–Hc] at 335 nm (Nickerson and Van Holde, 1971), under each of the four conditions. The data were analyzed as paired observations according to Student's t-test, using the 0.05 level as the criterion of significance.

Subunit composition

Polyacrylamide gel electrophoresis was performed according to the method of Davis (1964), using slabs of 7.5% acrylamide gel (1.5 mm thick), and 25–30 μg Hc, stripped by dialysis against 0.05 *M* Tris buffer containing 0.01 *M* EDTA (pH 8.9).

Results

The effect of experimental design

As indicated above, the experiments on the effects of inorganic ions on HcO_2 binding at a common pH were designed so that each point connected by the curves in Figures 1 and 2 represents a different pool of blood, of necessity taken from animals collected during different seasons and, possibly, from different salinities in the range about 0–25‰. In view of the seasonal changes in HcO_2 affinity demonstrated in this species earlier (Mauro and Mangum, 1982) and the effects of acclimation salinity demonstrated below, appreciable scatter around the curves would be expected. In contrast, the experiments on the effects of inorganic ions on the Bohr shift were designed so that the points connected by the curves in Figures 3–6 were obtained from a single pool of blood. Considerably less scatter around the curves would be expected. These expectations are realized in the results.

Since acclimation does not appear to influence cooperativity in this species, the error around the values in Tables II and III should be unrelated to experimental design.

TABLE II

The effect of inorganic ions on cooperativity (n_{50}) of HcO_2 binding in C. sapidus *Hc*[1]

Solution	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Distilled water	0	2.9 ± 0.2
Complete salines	194	3.0 ± 0.3
	386	3.2 ± 0.4
	574	3.4 ± 0.4
	764	2.9 ± 0.2
Single salts:		
NaCl	90	3.2 ± 0.2
	183	2.8 ± 0.2
	270	2.7 ± 0.3
	359	3.0 ± 0.3
KCl	2.5	2.6 ± 0.8
	5.0	1.7 ± 0.9
	7.5	2.5 ± 0.2
	10.0	2.6 ± 0.3
$CaCl_2$	2.6	2.5 ± 0.5
	4.8	3.6 ± 0.4
	7.0	3.1 ± 0.4
	9.2	2.8 ± 0.3
$MgCl_2$	1.1	2.9 ± 0.2
	1.8	3.3 ± 0.2
	2.5	3.2 ± 0.6
	3.3	3.8 ± 0.6
	9.2	3.4 ± 0.4
$Ca(NO_3)_2$	7.0	2.8 ± 0.4
Salt mixtures:		
$NaCl/MgCl_2$	90/1.1	3.3 ± 0.2
	183/1.8	3.8 ± 0.3
	270/2.5	3.1 ± 0.2
	359/3.3	3.6 ± 0.2
$NaCl/CaCl_2$	90/2.6	3.3 ± 0.2
	183/4.8	3.8 ± 0.4
	270/7.0	3.1 ± 0.3
	359/9.2	3.7 ± 0.2
$CaCl_2/MgCl_2$	2.6/1.1	3.3 ± 0.2
	4.8/1.8	3.7 ± 0.4
	7.0/2.5	3.1 ± 0.6
	9.2/3.3	2.8 ± 0.3
$NaCl/MgCl_2/CaCl_2$	90/1.1/2.6	3.6 ± 1.8
	183/1.8/4.8	3.6 ± 0.5
	270/2.5/7.0	3.1 ± 0.3
	359/3.3/9.2	2.9 ± 0.6
$NaNO_3/Mg(NO_3)_2/Ca(NO_3)_2$	270/2.5/7.0	2.8 ± 0.2

[1] 0.05 *M* Tris Maleate, 25°C, pH 7.48–7.52. N = 2–4.

The effect of total salinity

When the preparation is dialyzed against buffered distilled water, HcO_2 affinity becomes very low (Figs. 1 and 2), but cooperativity remains unchanged (Table II). When the preparation is dialyzed against a physiological saline containing all of the major ions found in the blood, HcO_2 affinity increases with an increase in total salt concentration (Figs. 1 and 2). Cooperativity, however, does not change (Table II).

The effect of single salts

At activities similar to those in the complete salines, no single inorganic salt present by itself clearly raises HcO_2 affinity in full to the level observed in the complete salines (Fig. 1). In the presence of small amounts of buffered KCl, the response does not differ significantly from that observed after dialysis against buffered distilled water. The presence of buffered NaCl (or $MgCl_2$) does increase HcO_2 affinity but, above 90 meq free Na^+/l, P_{50} appears to be independent of NaCl concentration. By far the most important single salt is $CaCl_2$, which very nearly restores HcO_2 affinity to the control levels. Indeed, the difference between the data for $CaCl_2$ and for the complete physiological saline, although significant, is very small. Ca^{+2} has a specific effect, an inference supported by the value at 9.2 meq/l $MgCl_2$; at this level, the free Mg^{+2} concentration approximates the highest free Ca^{+2} concentration used. In addition, Mg^{+2} has a much smaller effect than Ca^{+2} at similar activities (Fig. 1). At virtually equal activities, there appears to be a difference between the effects of $CaCl_2$ and $Ca(NO_3)_2$, but it is very small.

Increasing the concentrations of single salts at pH 7.5 has no significant effect on cooperativity (Table II).

The effects of salt mixtures

In the presence of two or three salts, the responses of P_{50} are not always simple sums of the single salt values (Figs. 1 and 2). In the presence of NaCl and $MgCl_2$, HcO_2 affinity behaves very much as it does in the presence of NaCl alone (Fig. 2). Mixing NaCl with $CaCl_2$ appears to mitigate the effect of $CaCl_2$. The addition of $MgCl_2$ to either $CaCl_2$ or the mixture of NaCl and $CaCl_2$ produces a result virtually indistinguishable from that of the complete saline. In these results the replacement of Cl_2 with NO_3^- in the mixture of Na^+, Mg^{+2} and Ca^{+2} has no detectable effect on P_{50} (Fig. 2). Regardless of the mixture, n_{50} does not change (Table II).

The effects of inorganic ions on the Bohr shift

When the preparation is dialyzed against distilled water, the Bohr shift is virtually eliminated ($\Delta \log P_{50}/\Delta pH = -0.16$ in the pH range 6.97–8.07; Fig. 3). However, in the most dilute physiological saline, the Bohr shift is restored in full (the Bohr factor, $\Delta \log P_{50}/\Delta pH = -0.99$ in the pH range 6.95–8.03) and it does not increase further in more concentrated physiological salines (Fig. 3). While the Bohr factor appears to increase with $MgCl_2$ concentration (Fig. 4), it remains the same in the presence of various levels of $CaCl_2$ alone, or in a mixture of the two (Figs. 5 and 6).

In contrast to the small difference observed at pH 7.5 (Fig. 1), the more extensive observations in Figures 3 and 5 indicate that the presence of $CaCl_2$ alone restores HcO_2 affinity to the control level, at least in the range 7.0–9.2 meq free Ca^{+2}/l, and that the presence of Mg^{+2} is not necessary.

Although n_{50} generally appears to reach a maximum at about pH 7.8 (Table III),

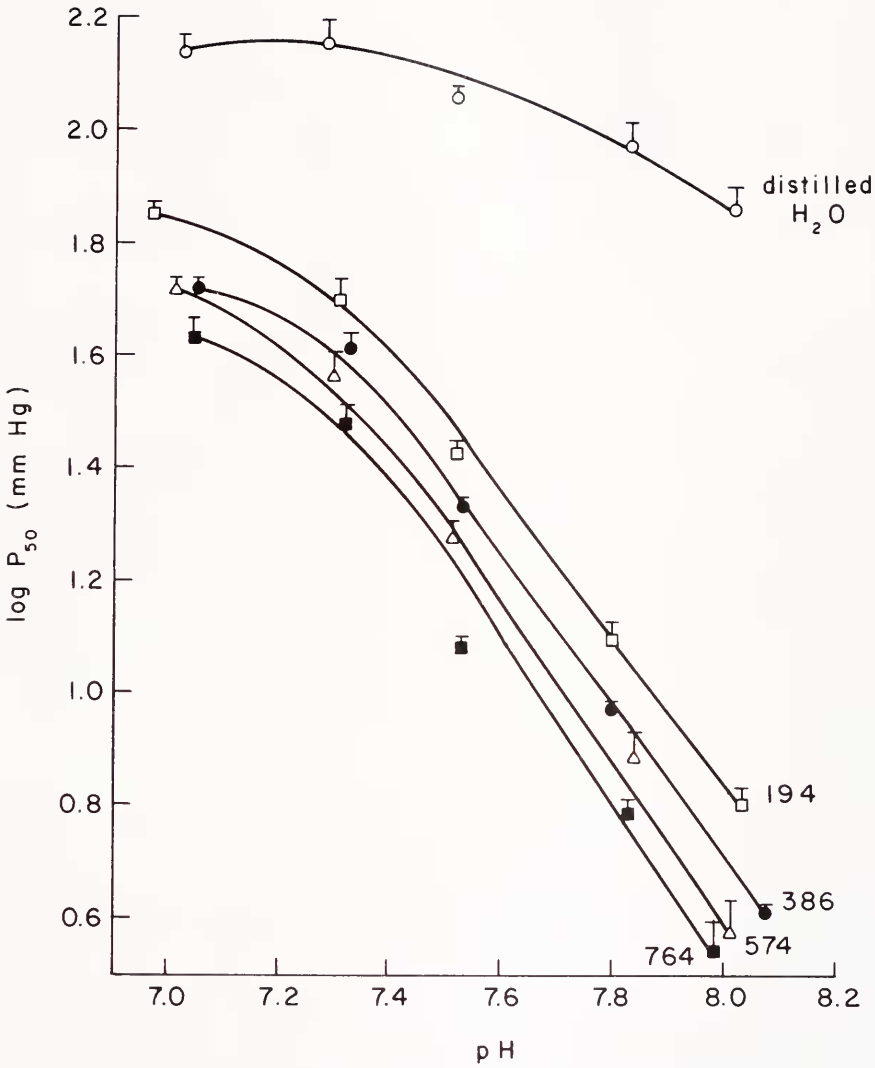

FIGURE 3. Effect of total salinity on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration. Bars show 95% confidence interval.

the trend is not significant, in part because of the small number of observations in each data set. Only in the presence of pure $MgCl_2$, where n_{50} continues to rise throughout the range examined, does pH significantly influence cooperativity (Table III).

The effect of salinity acclimation

Animals collected at high and low salinities have Hcs with significantly different O_2 affinities (Fig. 7). The high salinity population has a higher HcO_2 affinity than the low salinity population. The difference disappears completely 8 days after transfer of the low salinity crabs to high salinity, and it disappears in a large part 8 days after transfer of the high salinity crabs to low salinity. In the latter case, the trend

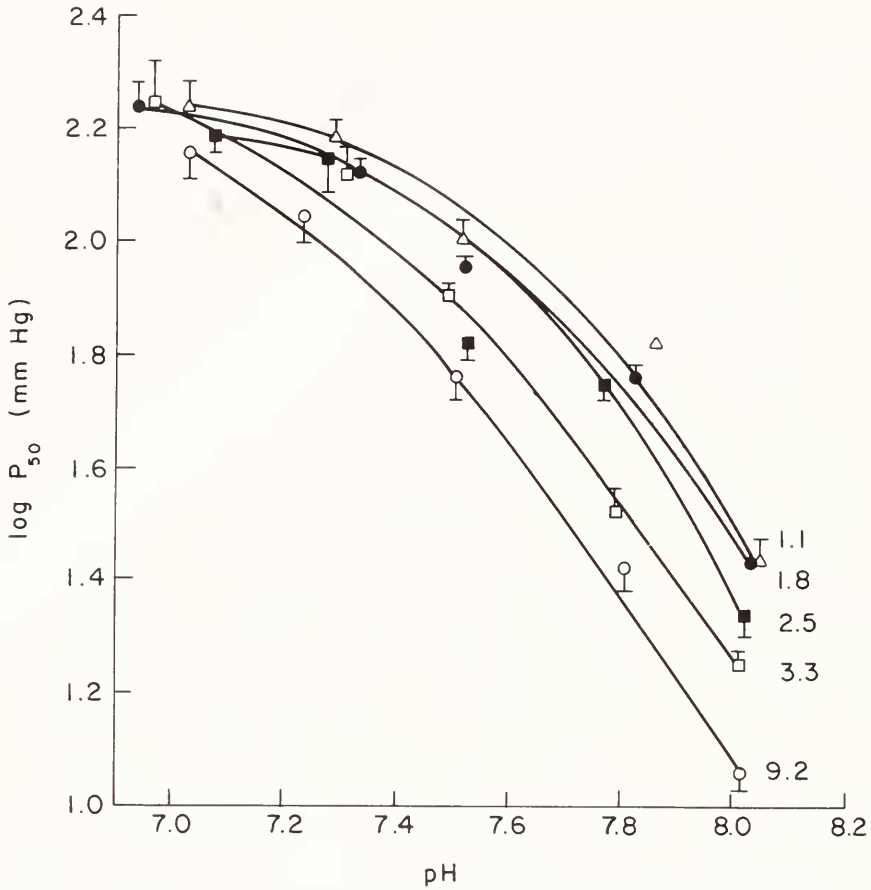

FIGURE 4. Effect of $MgCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration. Bars show 95% confidence interval.

may reflect individual differences since only one of the six points is significantly different. No change in cooperativity was detected (Table IV; Fig. 7).

The data in Table V suggest a change in Hc concentration, namely an increase at low salinity and a decrease at high salinity. However, the differences are significant only in the group transferred from high to low salinity.

Preliminary observations on the subunit composition of the hemocyanins suggest that, depending on resolution, as few as five or as many as seven electrophoretically separable polypeptides can be detected in both populations. Of 25 pools used in the investigation of inorganic ion effects, all obtained from Chesapeake Bay crabs, 23 are alike; they are also like a pooled preparation of the samples taken from the low salinity crabs used in the acclimation experiment, but they differ from a pool of the samples taken from the high salinity crabs used in the acclimation experiment.

Specifically, bands three and five are far more concentrated in the low than in the high salinity pool (Fig. 8). After making the oxygen binding measurements, material was available for electrophoresis only from the group transferred from low

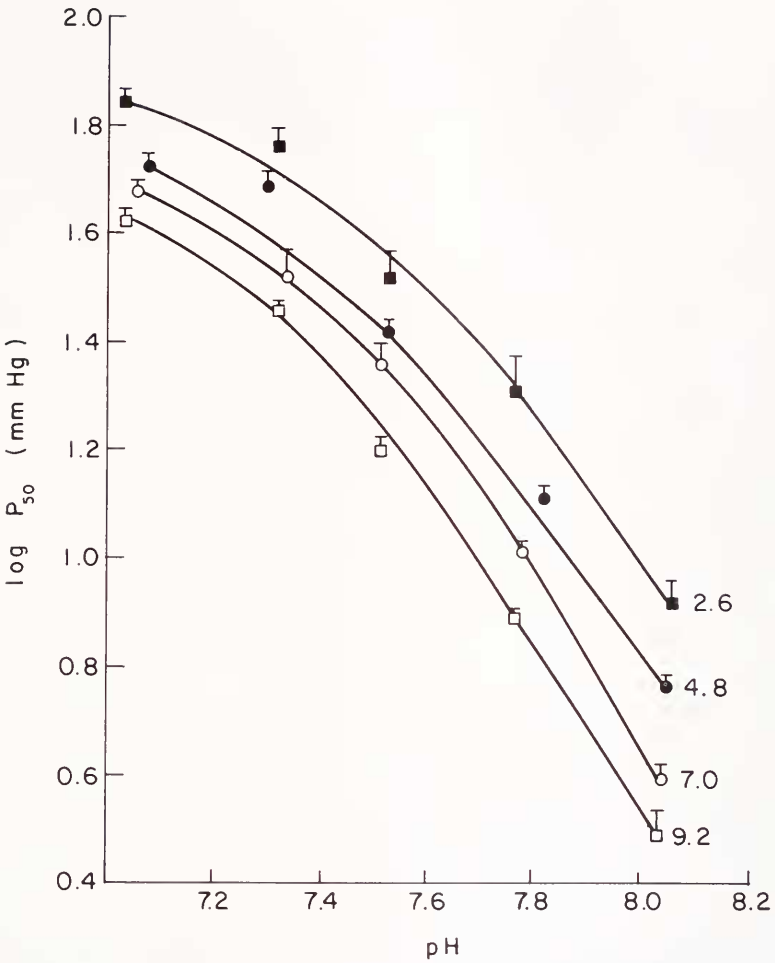

FIGURE 5. Effect of $CaCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentrations. Bars show 95% confidence interval.

to high salinity. Perhaps more interesting, this pool was no longer exactly like the one obtained prior to the transfer; instead, bands three and five had become less concentrated, the pattern appearing intermediate between the two populations (Fig. 8). This observation was made in replicate.

DISCUSSION

The labile oxygenation properties and their inorganic effectors

The O_2 affinity of *C. sapidus* Hc rises measurably when the total salinity increases within the physiological range. The levels of K^+ in the blood are apparently too small to have a detectable effect, and the effects of Na^+ are moderate, reaching their maximum either at or below 90 meq free Na^+/l. Increases in Ca^{+2} and Mg^{+2}, on the other hand, continue to raise HcO_2 affinity throughout the physiological range.

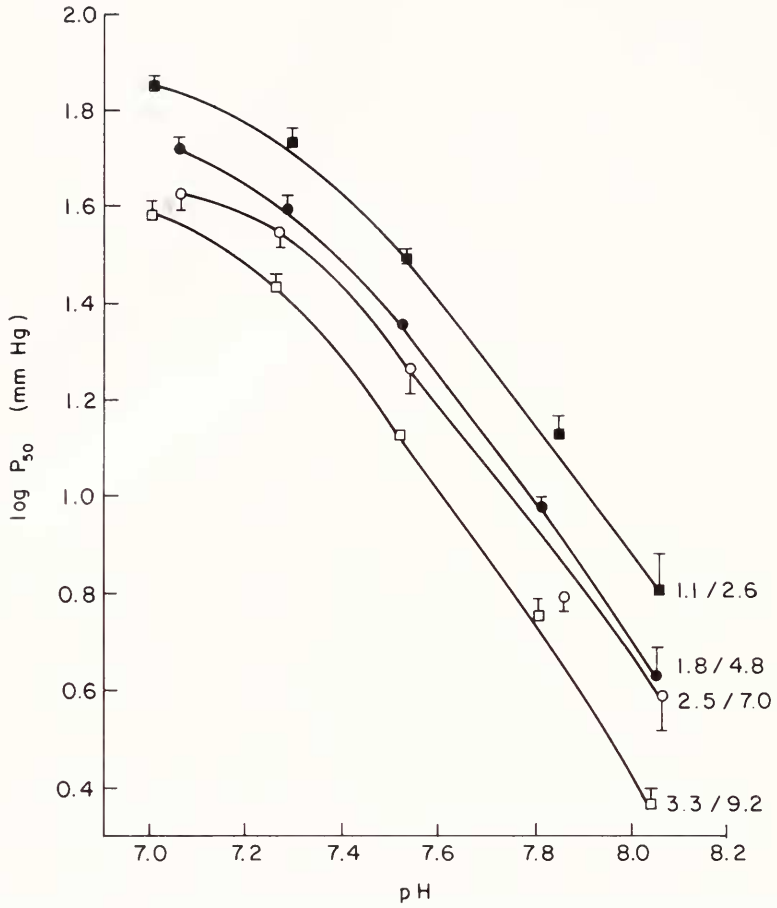

FIGURE 6. Effect of a mixture of $MgCl_2$ and $CaCl_2$ on the Bohr shift of *C. sapidus* Hc in 0.05 *M* Tris Maleate at 25°C. Curves are designated by free ion concentration, with $MgCl_2$ given first. Bars show 95% confidence interval.

Of the two, Ca^{+2} is clearly the more important, by a factor approaching six. A factor describing Ca^{+2} sensitivity ($\Delta \log P_{50}/\Delta \log [Ca^{+2}]$) is almost three times greater in *C. sapidus* (−0.82; Fig. 5) than in *C. maenas* (−0.28; Truchot, 1975). Indeed, our data (Figures 3 and 5) suggest that, as long as free Ca^{+2} exceeds 7 meq/l, no other inorganic ion is required to restore HcO_2 affinity to the level found in a complete physiological saline.

While the effect of Ca^{+2} is very large and highly specific, a specific Cl^- effect is either absent or so small that it cannot be clearly discerned. The magnitude of the change in Hc molecular weight following the removal of Ca^{+2} at physiological pH (7.8) (Herskovits *et al.*, 1981) suggests that little, if any, of the Ca^{+2} effect results from dissociation of the native dodecamer. The absence of a Ca^{+2} effect on cooperativity also supports this conclusion.

Using chelating agents to remove divalent cations, other investigators (Chantler *et al.*, 1973) have reported an effect of Ca^{+2} on the cooperativity of portunid crab

TABLE III

The effect of inorganic ions and pH on cooperativity (n_{50}) of HcO_2 binding in C. sapidus[1]

Sample	Free ion concentration (meq/l)	pH (±0.02)	n_{50} (mean ± S.E.)
Distilled water	0	7.01	2.2 ± 0.3
		7.27	2.3 ± 1.0
		7.51	3.0 ± 0.2
		7.82	2.6 ± 0.3
		8.00	3.3 ± 0.8
Complete salines	194	6.97	2.6 ± 0.3
		7.30	2.8 ± 0.5
		7.49	3.0 ± 0.3
		7.79	3.9 ± 0.9
		8.03	3.1 ± 0.3
	386	7.04	2.6 ± 0.6
		7.30	2.8 ± 0.8
		7.49	3.2 ± 0.4
		7.79	3.6 ± 0.5
		8.06	2.3 ± 0.8
	574	7.00	2.7 ± 0.3
		7.29	2.8 ± 0.7
		7.50	3.4 ± 0.2
		7.83	3.5 ± 0.6
		8.01	2.8 ± 0.8
	764	7.03	3.0 ± 0.7
		7.32	3.1 ± 0.5
		7.52	3.0 ± 0.2
		7.82	3.8 ± 0.6
		7.98	2.7 ± 0.8
$CaCl_2$	2.6	7.02	2.9 ± 0.2
		7.31	2.7 ± 0.4
		7.52	2.5 ± 0.6
		7.76	2.8 ± 0.5
		8.05	2.7 ± 0.9
	4.8	7.06	2.6 ± 0.9
		7.29	3.2 ± 0.6
		7.49	3.6 ± 0.4
		7.81	3.4 ± 0.3
		8.04	3.8 ± 0.6
$CaCl_2$	7.0	7.05	2.8 ± 0.3
		7.33	2.2 ± 0.8
		7.49	3.1 ± 0.4
		7.78	3.1 ± 0.4
		8.04	2.6 ± 0.6
	9.2	7.03	2.5 ± 0.2
		7.31	3.1 ± 0.3
		7.50	2.8 ± 0.3
		7.76	3.2 ± 0.2
		8.03	2.9 ± 0.4

TABLE III (*Continued*)

Sample	Free ion concentration (meq/l)	pH (±0.02)	n_{50} (mean ± S.E.)
$MgCl_2$	1.1	7.02	1.8 ± 0.3
		7.28	2.2 ± 0.2
		7.50	2.9 ± 0.2
		7.85	3.9 ± 0.3
		8.05	3.4 ± 0.8
	1.8	6.95	1.9 ± 0.4
		7.32	2.5 ± 0.3
		7.48	3.3 ± 0.2
		7.82	3.9 ± 0.3
		8.03	3.8 ± 0.4
	2.5	7.06	2.2 ± 0.2
		7.27	2.4 ± 0.6
		7.52	3.2 ± 0.4
		7.77	4.0 ± 0.4
		8.02	4.1 ± 0.5
	3.3	6.96	2.0 ± 0.6
		7.31	2.7 ± 0.6
		7.50	3.5 ± 0.4
		7.79	4.0 ± 0.3
		8.01	4.5 ± 0.6
	9.2	7.02	2.3 ± 0.5
		7.24	3.2 ± 0.4
		7.50	3.8 ± 0.7
		7.80	4.2 ± 0.4
		8.01	4.8 ± 0.5
$MgCl_2/CaCl_2$	1.1/2.6	7.00	3.0 ± 0.2
		7.28	2.8 ± 0.3
		7.49	3.3 ± 0.2
		7.84	3.5 ± 1.1
		8.05	2.6 ± 0.7
$MgCl_2/CaCl_2$	1.8/4.8	7.05	3.2 ± 0.2
		7.28	2.8 ± 0.6
		7.50	3.7 ± 0.4
		7.80	3.3 ± 0.4
		8.04	2.4 ± 0.8
	2.5/7.0	7.05	2.5 ± 0.3
		7.25	2.6 ± 0.3
		7.49	3.1 ± 0.6
		7.85	3.9 ± 0.8
		8.06	2.5 ± 1.0
	3.3/9.2	6.99	3.2 ± 0.4
		7.26	3.1 ± 0.5
		7.51	2.8 ± 0.3
		7.81	3.0 ± 0.9
		8.04	2.5 ± 0.9

[1] 0.05 *M* Tris Maleate, 25°C. N = 2–4.

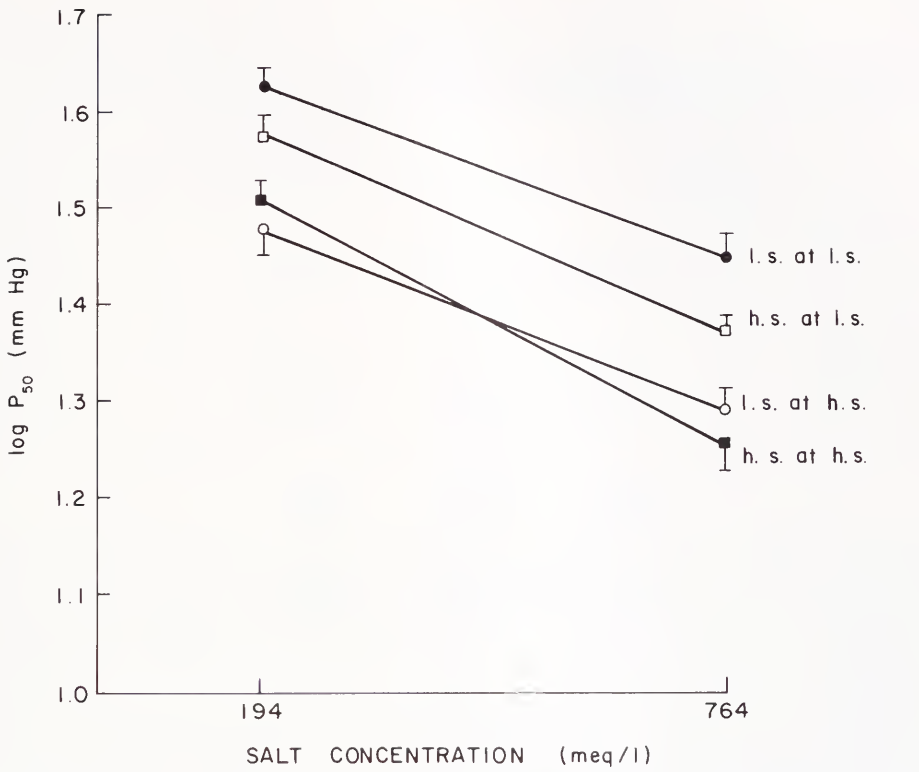

FIGURE 7. The effect of acclimation salinity on O_2 affinity of *C. sapidus* Hc (0.05 *M* Tris Maleate) at 25°C and pH 7.53 ± 0.02. Low salinity (0–3‰) population (l.s. at l.s.), low salinity population after 8 days at 35‰ (l.s. at h.s.), high salinity (34‰) population (h.s. at h.s.), and high salinity population after 8 days at 5–8‰ (h.s. at l.s.). Blood was dialyzed against 194 and 764 meq/l complete physiological saline solutions. Paired observations were made ($N = 6$ for the low salinity population, $N = 7$ for the high salinity population). Bars show 95% confidence interval.

HcO_2 binding with the physiological pH range (6.9 to 8.1). At pH 10, the removal of Mg^{+2} causes complete dissociation to monomers (Hamlin and Fish, 1977), which would eliminate cooperativity. However, the present findings clearly indicate that changes of orders of magnitude in excess of the physiological range are necessary to influence cooperativity in *C. sapidus*. The present results also indicate that very little Ca^{+2} is required to maintain the Bohr shift at the physiological level, even less than the amount needed to restore HcO_2 affinity. Thus, HcO_2 affinity is the only oxygenation property that responds to changes in the inorganic ions in the blood.

The role of the unidentified effector

Although highly variable, the acute measurements of blood calcium made on freshly collected crabs by Colvocoresses *et al.* (1974) suggest that the average value at 0‰ would be about 27 meq/l, and the average value at 35‰ would be about 34 meq/l. At 27 meq total Ca/l, 1 (= *ca.* 5.3 m*M* free Ca^{+2}/l) P_{50} would be about 27 mm Hg (pH 7.5; Fig. 1); at 34 meq total Ca^{+2}/l (= 6.8 m*M* free Ca^{+2}/l), P_{50} would be 23 mm Hg. The average changes in Ca^{+2} would alter HcO_2 by 4 mm Hg; hence

TABLE IV

Cooperativity (n_{50}) of HcO_2 binding in native and acclimated C. sapidus *populations after dialysis against 194 meq/l or 764 meq/l complete physiological salines*[1]

Sample	Free ion concentration (meq/l)	n_{50} (mean ± S.E.)
Low salinity population (0–3‰)	194	3.0 ± 0.2
(N = 6)	764	3.5 ± 0.3
Low salinity population after 8 days at 35‰	194	3.4 ± 0.5
(N =6)	764	3.2 ± 0.9
High salinity population (34‰)	194	3.3 ± 0.5
(N = 7)	764	3.4 ± 0.3
High salinity population after 8 days at 5–8‰	194	3.8 ± 0.7
(N = 7)	764	3.3 ± 0.4

[1] 0.05 *M* Tris Maleate (pH 7.51–7.55), 25°C.

the direct effect of total salinity on the blood of an individual observed by Mangum and Towle (1977). However, the physiological response of the HcO_2 transport system is far more complicated than previously supposed. At pH 7.5 and low salt concentration the actual difference in HcO_2 affinity between populations acclimated to normoxic water and to salinities only slightly less different than 0 and 35‰ is about 43–32 = 11 mm Hg (Fig. 7); at high salt concentration, the difference is about the same (29–18.5 = 10.5 mm Hg). Thus the effect of low salinity acclimation on HcO_2 affinity is considerably larger than that attributed by Mangum and Towle (1977) to the salt effect alone. The increment is due to changes in a non-dialyzable factor which are induced, in large part or in full, within 8 days.

Because the data were obtained from dialyzed preparations, the most probable explanation of the acclimation might seem to be a change in the protein *per se*, resulting in Hcs with intrinsically different O_2 affinities in the high and low acclimation states. Two lines of evidence might be invoked to support this suggestion: the increase in total hemocyanin concentration and the increase in concentration of two of the 5–7 subunits at low salinity. In our view, however, neither set of evidence is particularly cogent.

Using unpaired observations on different individuals held at high and low salinity, a number of investigators have examined the relationship in portunid crabs between environmental salinity and either total protein concentration (Horn and Kerr, 1963; Lynch and Webb, 1973; Péqueux *et al.*, 1979) or Hc concentration

TABLE V

The effect of acclimation salinity on Hc concentration (mean ± S.D.) in C. sapidus[1]

Sample	Hc concentration (g/100 ml)
Low salinity population (0–3‰) (N = 6)	5.9 ± 1.8
Change in low salinity population after 8 days at 35‰ (N = 6)	−3.2 ± 2.3
High salinity population (34‰) (N = 7)	6.1 ± 1.3
Change in high salinity population after 8 days at 5–8‰ (N = 7)	+2.2 ± 0.8

[1] Paired observations on the same individuals before and after transfer to the alternative salinity.

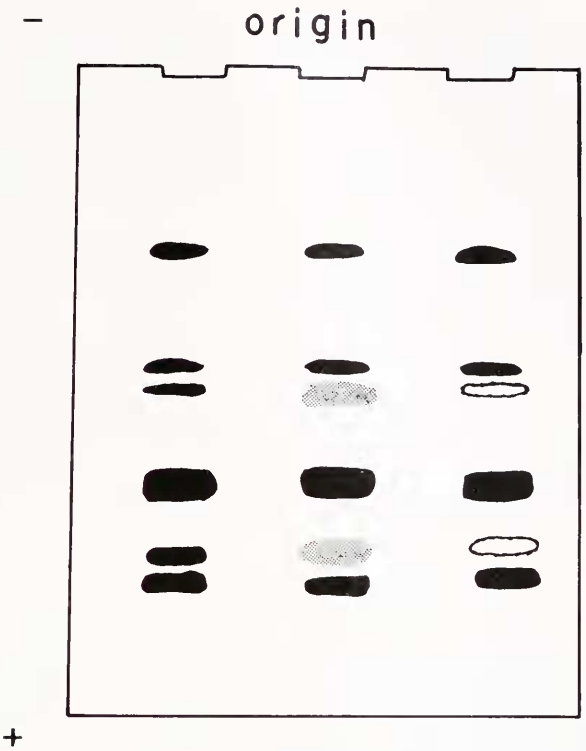

FIGURE 8. The effect of acclimation salinity on the concentration of electrophoretically separable polypeptides of *C. sapidus* Hc. Left to right: Low salinity population, low salinity population after 8 days at 35‰, and high salinity population.

(Boone and Schoffeniels, 1979). Boone and Schoffeniels (1979) and Péqueux *et al.* (1979) both reported extremely large, though not significant, changes that could be invoked to support an hypothesis of *de novo* Hc synthesis at low salinity. Horn and Kerr (1963) mention no clear relationship between salinity and either protein or Cu concentration, and the more numerous observations on serum protein in freshly collected animals also show no clear trend (Lynch and Webb, 1973). The enormous variation among different individuals may preclude a very firm conclusion from the previous investigations.

Our own data, based on paired observations following transfers in both directions and thus eliminating individual variation as well as differences in nutritional state, indicate that a net increase in synthesis, if it occurs at all, is smaller than reported previously (Péqueux *et al.*, 1979). Since blood volume remains essentially constant over a wide salinity range (Robinson, 1982), a net change in Hc synthesis would not be masked by concomitant changes in extracellular space. On the other hand, it is likely that an increase in synthesis would be masked by a concomitant increase in degradation to keep the Hc concentration from exceeding a level that can be efficiently handled by the cardiovascular system (Snyder and Mangum, 1982). Thus the available information on Hc concentration argues neither for nor against the replacement of one Hc molecule by another as the mechanism of low salinity acclimation.

Although the role of subunit heterogeneity in assembling the hemocyanin poly-

mers is presently becoming clear (*e.g.* Markl and Kempter, 1981), its physiological significance, if any, has yet to be investigated. The shift in subunit ratios following acclimation of *C. sapidus* to a new salinity is certainly an interesting observation, the meaning of which will be pursued by determining the frequencies of the two phenotypes in the high and low salinity populations, and by correlating the shift with HcO_2 affinity on an individual basis. However, we should point out that the shift, which was incomplete, was observed in the group in which the acclimation of HcO_2 affinity was complete.

Regardless of the mechanism, the significance of the salinity acclimation is very clear. In the laboratory, brief (8 h) exposure to hypoxic water results in large increases (>5 m*M*) in blood lactate (Mangum, unpublished data). If unopposed by concomitant changes in other effectors of HcO_2 binding, these levels could raise HcO_2 affinity by a factor large enough to impair O_2 delivery to the tissues. In fact, the conjunction of pH, lactate, Ca^{+2} and salinity acclimation effects may prove to account for the smaller increase in HcO_2 affinity observed in freshly caught estuarine animals by Mangum and Towle (1977) and attributed to inorganic ions alone. The actual balance achieved by the various effectors of HcO_2 affinity awaits further investigation of animals acclimated to both dilute and moderately hypoxic water.

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